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Isolation of a novel complement regulatory factor (GCRF) from glomerular epithelial cells

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Isolation of a novel complement regulatory factor (GCRF) from glomerular epithelial cells. Cultured rat glomerular epithelial cells (GEC) are able to prevent both antibody-directed and spontaneous (alternative pathway) complement activation. In this study, a novel complement regulatory factor (GCRF) was isolated from GEC. The ability to accelerate the decay of alternative pathway C3/C5 convertases formed on sheep erythrocytes (EC3bBbP) was used to guide purification. GEC were solubilized in Triton X-114 and GCRF was recovered in the aqueous phase. Complement inhibitory material also was present in the culture supernatant, which likely represented GCRF. By Mono Q anion exchange chromatography, GCRF eluted at $\geq$ 0.6 M NaCl and by Superose 6 size-exclusion chromatography, it had a $K_{av} \leq 0.3$. GCRF reduced the $t_{1/2}$ of EC3bBbP from 128 minutes in buffer alone to 41 minutes in 3 μg/ml GCRF protein, and also prevented formation of EC3bBbP in a dose-dependent fashion. Digestion with chondroitinase ABC, neuraminidase, or trypsin, but not with heparitinase or chondroitinase AC significantly reduced the activity and size of GCRF, demonstrating that it is a sialic acid-containing dermatan sulfate proteoglycan. Thus, cultured rat GEC synthesize and secrete into the medium, GCRF, a dermatan sulfate proteoglycan with complement inhibitory activity.

Complement activation is regulated at several steps throughout the cascades of the alternative and classical pathways. The list of proteins that serve these regulatory functions continues to grow. At the level of the C3 and C5 convertases, these are the fluid phase proteins, factor H [1] and C4 binding protein [2], and the cell membrane proteins, C3b receptor (CR1) [3], decay accelerating factor (DAF) [4], and membrane cofactor protein [5]. With the exception of membrane cofactor protein, all accelerate the decay of alternative and/or classical pathway C3 and C5 convertases [6].

We previously have shown that cultured human glomerular epithelial cells (GEC) express DAF on their surface which limits antibody-directed complement activation [7]. We have also demonstrated that cultured rat GEC have complement inhibitory activity on their surface that is susceptible to proteolytic enzyme digestion. The pattern of enzyme sensitivity was consistent with known enzyme sensitivities of human DAF, suggesting that rat GEC also express DAF, or a related protein on their membrane [7]. Human GEC also express CR1 [8], and Kasinath et al recently have identified this protein on the surface of cultured rat GEC [9]. Hence, CR1 may also play a role in limiting complement activation on GEC.

The glomerular capillary wall, composed of a fenestrated endothelial cell, the glomerular basement membrane, and the GEC, functions as a size- and charge-restrictive filter of plasma to form a largely protein-free urine [10]. An important component of this filter are negatively charged proteoglycans composed of a protein core and attached glycosaminoglycans, heparan sulfate and chondroitin sulfates [11, 12]. GEC are believed to be the major source of proteoglycans [13], which is supported by recent studies showing that cultured human and mouse GEC synthesize heparan and chondroitin sulfate proteoglycans and secrete them into the medium [14–16]. Besides their role in maintaining the integrity of the glomerular capillary wall, the function of glomerular proteoglycans is largely unknown, although proteoglycans from other sites have been shown to have a role in cell adhesion and in cell differentiation [17]. In this study, the predominant complement inhibitory material produced by cultured rat GEC was a dermatan sulfate proteoglycan, and not DAF or CR1. This proteoglycan, termed GCRF, may limit complement activation in the glomerular capillary wall and on the GEC surface in vivo.

Methods

Materials

Buffers used in hemolytic assays were: veronal buffered saline (VBS, 145 mM NaCl, 5 mM Na barbital, pH 7.4); half isotonic VBS containing 2.5% dextrose, 0.1% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$ ($DGVB^{++}$); and, VBS containing 0.1% gelatin and 10 mM EDTA (GVB-EDTA).

Chondroitinase ABC, chondroitinase AC, heparitinase, *Streptomyces* hyaluronidase, neuraminidase (Type V), trypsin, pronase (type XIV protease), Triton X-114, soybean trypsin inhibitor, benzamidine, diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), ϵ-amino-n-caproic acid, iodoacetamide, guanidine HCl, chondroitin sulfates A, B and C, and heparan sulfate were from Sigma Chemical Co. (St. Louis, Missouri, USA). Chondroitin sulfate B was from bovine mucosa and contained $\approx$ 85% chondroitin sulfate B (dermatan sulfate), the remainder being types A and C. [^{35}S]sulfate (43 Ci/mg S, carrier free) was from ICN Biomedicals (Costa Mesa, California, USA) and [4,5-^{3}H]leucine (53 Ci/mmol) was from New England Nuclear (Boston, Massachusetts, USA). Bio-Rex 70 was from Bio-Rad Laboratories (Richmond, California,

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USA), and Sephadex G-25 and G-50 (fine), Sepharose CL-4B, and prepacked columns of Mono Q and Superose 6 were from Pharmacia, Inc. (Piscattaway, New Jersey, USA).

Human C3 [18] and factor B [19] were purified according to published methods. Human factors D and P were purchased from Quidel (San Diego, California, USA). Rat C3 was purified according to the method of Vranian, Conrad and Ruddy [20] with minor modifications. Ninety ml of normal rat serum (NRS) was fractionated with 5 to 12% polyethylene glycol and then sequentially chromatographed on L-lysine-Sepharose, DEAE-Sephacel, Sepharose CL-6B, and Sephacryl S-200 (Pharmacia). C3 was purified 35-fold with a yield of 29.2 mg as determined by radial immunodiffusion. C3 activity was 93,000 U/mg, while the activity of C5 (the protein most likely to copurify with C3 [18]) was 3 U/mg.

GEC culture

Rat GEC were cultured on a collagen matrix (Vitrogen, Collagen Corp., Palo Alto, California, USA) in K1 medium, which is Dulbecco's Modified Eagle Medium (DMEM)/Ham's Nutrient Mixture F-10 (1:1) (GIBCO, Grand Island, New York, USA), containing 5% Nu-Serum (Collaborative Research, Bedford, Massachusetts, USA) and hormone supplements [21]. These cells have been previously characterized [21] and were used between passages 13 and 35. Once cells were confluent in 100 mm culture dishes, the medium was removed, the cells were washed twice with phosphate-buffered saline, pH 7.2 (PBS), and then removed with the adherent collagen, which was digested with 0.1% collagenase (Type V, Sigma) for 30 minutes at 37°C, following which GEC were washed extensively with PBS. It should be noted that collagenase was necessary given the collagen requirements of the GEC line, and the large volume which the collagen occupies (3 to 4 ml per culture dish). It is possible that cell surface proteins were digested by contaminating proteases within the collagenase preparation, however, as the cells remained viable during collagenase treatment, intracellular proteins should not have been affected.

For the majority of experiments, cells were maintained in K1 medium until isolation. In one experiment, to evaluate complement inhibitory activity in the medium, nearly confluent cells in five 100 mm culture dishes were cultured for 40 hours in medium consisting of DMEM/Ham's F-10 (1:1) with 0.4% fetal calf serum, following which the conditioned medium was harvested and any free cells removed by centrifugation. GEC remained viable during this period, however, cell division was markedly reduced [22].

Solubilization of GEC

Triton X-114 was used to solubilize GEC, because of its low cloud point temperature [23]. With phase separation, the bulk of proteins segregate into the aqueous phase, whereas integral membrane proteins [23] and proteins linked via phosphatidylinositol to the plasma membrane, such as DAF [24, 25], are recovered in the lipid phase. The GEC pellet was solubilized in 100 mM NaCl, 20 mM bis-Tris, pH 6.0 (bis-Tris buffer) containing 10 mM EDTA, 10 mM iodoacetamide, 5 mM DFP, 1% Triton X-114 (0.2 ml per culture dish) for 15 minutes on ice, followed by centrifugation at 3750 rpm for 10 minutes at 4°C. The supernatant was then warmed to 22°C (above the cloud point of Triton X-114 [23]), and centrifuged for 10 minutes at 15,000 rpm. The aqueous phase appearing in the upper layer was removed, and the lipid-rich pellet was reconstituted with the solubilizing buffer without Triton X-114, and both were placed on ice. From three separate isolations from 50 to 100 culture dishes, the total recovery of protein (determined by the Pierce BCA assay, Rockford, Illinois, USA) was 1.06 ± 0.35 mg per 100 mm culture dish (mean ± SD) with 87.9 ± 0.7% of protein recovered in the aqueous phase.

Determination of complement inhibitory activity

The means to assess complement inhibitory activity was based on the ability of collected fractions to decay complement intermediates formed on sheep erythrocytes (E), as has been used in the past to identify and purify complement regulatory proteins [1, 3, 4]. In order to form alternative pathway C3/C5 convertases, rat C3b was bound via its free sulfhydryl group [26] to sheep E with a heterobifunctional cross linking agent. E at 2×10^9/ml in VBS were incubated with 2 mM sulfosuccinimidyl (4-iodoacetyl) aminobenzoate (sulfo-SIAB, Pierce) for 30 minutes at 37°C. C3b was generated from rat C3 (in some experiments, where indicated, human C3 was used in an identical fashion) by incubating C3 with trypsin at a 100:1 ratio (wt:wt) for one minute at 37°C, following which a twofold molar excess of soybean trypsin inhibitor was added. After washing, E were resuspended at 4×10^9/ml and 500 μg/ml C3b was added for 60 minutes at 37°C to form EC3b. Cells were then adjusted to 5×10^7/ml, and sufficient B, D, and P were added for nine minutes at 30°C to form EC3bBbP, such that if convertases were decayed for 20 minutes in $DGVB^{++}$ alone there was an average of one hemolytic site per cell (z) [4], unless otherwise noted. Cells were washed in $DGVB^{++}$ and resuspended to 1×10^8/ml. One hundred μl of EC3bBbP was then added to an equal volume of $DGVB^{++}$ containing a 1/50 dilution of fractions to be assessed for inhibitory activity. After 20 minutes at 30°C, residual convertases were completed by addition of C3-C9 (300 μl of a 1/20 dilution of NRS in GVB-EDTA; NRS-EDTA) for 60 minutes at 37°C, and hemolysis determined. The capacity of the samples to reduce hemolysis (that is, accelerate decay of EC3bBbP) compared to buffer alone was calculated and expressed as percent z reduction [4].

The complement inhibitory effects of GCRF were characterized as follows. The time course of EC3bBbP inactivation was determined by incubating EC3bBbP at 1×10^8/ml in $DGVB^{++}$ with an equal volume of various concentrations of GCRF, or buffer alone. After various times, 200 μl of cells were removed and added to 300 μl NRS-EDTA for 60 minutes at 37°C, and hemolysis determined. To determine whether GCRF could affect EC3b prior to the formation of the alternative pathway C3/C5 convertase (that is, before addition of B, D, and P), EC3b at 1×10^8/ml were mixed with equal volumes of various concentrations of GCRF for 30 minutes at 30°C. Samples were then either used immediately or they were washed and resuspended to 5×10^7/ml, and B, D, and P added for nine minutes at 30°C. The resultant EC3bBbP were then completed with NRS-EDTA as before and hemolysis quantitated.

Studies with enzymes

To determine the nature of GCRF, digestion with different enzymes were performed, followed by assessment of functional activity and size. Seventy μl of GCRF was incubated with an

equal volume of chondroitinase ABC (1 U/ml), chondroitinase AC (1 U/ml), *Streptomyces* hyaluronidase (100 U/ml), trypsin (1 mg/ml) in 50 mM NaCl, 50 mM Tris, pH 7.0, or heparitinase (5 U/ml) or neuraminidase (1 U/ml) in 100 mM Na acetate, 10 mM $CaCl_2$, pH 7.0 for one hour at 37°C. In all incubations, except for with trypsin, DFP was added to 5 mM. At the end of the incubation, a twofold molar excess of soybean trypsin inhibitor was added to the tube containing trypsin. The resulting digests were then diluted 1/25 in $DGVB^{++}$ and 100 μl added to 100 μl EC3bBbP (1×10^8/ml). For these studies, human C3b was used and the input of B, D, and P were adjusted so that after a 90 minute decay at 30°C in untreated GCRF (that is, exposed to buffer alone), followed by addition of NRS-EDTA, z was 1.

In the case of chondroitinase ABC and neuraminidase, which had an effect on GCRF (**Results**), additional digestions were performed identically to those described above, however, in the presence of 10 mM benzamidine, 100 mM ϵ-amino-n-caproic acid, 10 mM iodoacetamide, 10 mM EDTA (chondroitinase ABC only), and 5 mM DFP in an attempt to neutralize any unrecognized protease activity in the enzyme preparations. To exclude a direct effect of the enzyme on the assay, EC3b at 1×10^8/ml were incubated for 90 minutes at 30°C with an equal volume of a 1/25 dilution of 0.5 U/ml chondroitinase ABC or neuraminidase in the presence of the protease inhibitors. They were then washed with $DGVB^{++}$ and made EC3bBbP with B, D, and P, as before. After washing, they were resuspended to 1×10^8/ml in $DGVB^{++}$ and decayed for 90 minutes at 30°C in an equal volume of GCRF or buffer alone, following which residual convertase sites were developed with NRS-EDTA.

The effect of enzymes on the size of GCRF was assessed by size-exclusion chromatography on a Superose 6 column run in 50 mM NaCl, 50 mM Tris, pH 7.0 using a Pharmacia FPLC system. Parallel determinations were done on GCRF incubated in enzyme inhibitors only.

Metabolic labelling of cells

GEC were labelled with [^{35}S]sulfate and [^{3}H]leucine as follows. All incubations were done at 37°C in a humidified atmosphere of 95% air/5% CO_2. Nearly confluent GEC in two 100 mm cultures dishes were washed twice with Hanks' balanced salt solution, and then incubated for three hours in K1 medium lacking leucine and $MgSO_4$, prepared using powdered DMEM/Ham's Nutrient mixture F-12 Base reconstituted with 2.5 mM glutamine, 0.5 mM lysine, 0.12 mM methionine (all from Sigma), 1.05 mM $CaCl_2$, and 0.7 mM $MgCl_2$, along with Nu-Serum which had been dialyzed against PBS. Cells were again washed, and then incubated for 15 hours in leucine- and $MgSO_4$-free K1 containing 25 μCi/ml [^{3}H]leucine and 100 μCi/ml [^{35}S]sulfate (4 ml/well). The medium was harvested, free cells removed by centrifugation, and the supernatant immediately placed on ice. Cells were washed three times with Hanks' balanced salt solution, and then incubated for 90 minutes in leucine- and $MgSO_4$-free K1 medium to which 1 mM unlabelled leucine and Na_2SO_4 had been added. Cells were washed, treated with collagenase, washed extensively with PBS, and then solubilized as described above. TCA precipitability of aqueous soluble ^{3}H- and ^{35}S-labelled material was 98.2 and 54.4%, respectively. Prior to use, both the medium and cell extract were passed over a Sephadex G-25 column equilibrated in bis-Tris buffer to remove free [^{35}S]sulfate and [^{3}H]leucine.

Size exclusion chromatography under dissociative conditions

The following studies were done in 4 M guanidine HCl, which dissociates non-covalently bound proteins [27]. Unlabelled GCRF was isolated as described in **Results**, and 500 μl applied to a Superose 6 column in 0.05 M Tris, 4 M guanidine HCl, pH 7.0 at 0.2 ml/min. One ml fractions were collected and 300 μl from each fraction was passed over a 3 ml column of Sephadex G-25 in $DGVB^{++}$. Collected fractions were diluted 1/3 and used in the hemolytic assay for complement inhibition.

Additional studies were performed on GEC that were labelled with [^{35}S]sulfate and solubilized in Triton X-114 as described above (unlabelled leucine was used in these experiments). The aqueous phase was made 4 M with solid guanidine and then exchanged into buffer consisting of 7 M urea, 0.02 M bis-Tris, 0.01 M EDTA, 0.1 mM PMSF, pH 6.0 by passage over a Sephadex G-50 column. ^{35}S-labelled GCRF was then isolated as described in **Results** and 200 μl was applied to a 118×0.9 cm column of Sepharose CL-4B in 4 M guanidine HCl, 0.1 M Na_2SO_4, 0.1 M Tris-HCl, 0.5% (vol/vol) Triton X-100 [28]. The column was run at 3 ml/hr and 1 ml fractions were collected and assessed for radioactivity. The void and total volumes of the column were determined with ^{3}H-labelled hyaluronic acid (provided by M. Yanagishita and J.C. Calvo, National Institute of Dental Research) and [^{35}S]sulfate, respectively. Additional labelled material was desalted on Sephadex G-25 in 0.05 M Tris-HCl, pH 7.2, for determination of complement inhibitory activity and for cellulose acetate electrophoresis.

Cellulose acetate electrophoresis

Glycosaminoglycans were isolated from GCRF either by digestion of the protein core with 10 μg/ml pronase for two hours at 50°C (for ^{35}S-labelled GCRF in 0.05 M Tris-HCl) or by β-elimination in 0.5 N NaOH for 24 hours at 4°C, followed by neutralization with HCl [27]. The resultant glycosaminoglycans were precipitated with 4 vols of absolute ethanol, centrifuged for 15 minutes at $8800 \times$ g, and the pellet resuspended in 1/5 vol water [29]. Cellulose acetate electrophoresis was performed using a Beckman Microzone Electrophoresis Cell (Palo Alto, California, USA) in 0.2 M calcium acetate, pH 7.0, for two hours at a constant current of 5 mA [29]. Five μl of GCRF glycosaminoglycans were run in parallel with standards (1.25 μg each). To determine the enzyme sensitivity of the GCRF glycosaminoglycans, incubations were performed in the presence of enzymes as described above. Cellulose acetate strips were stained with 0.1% alcian blue in 0.1% acetic acid, followed by destaining in 10% acetic acid [29]. In the case of ^{35}S-labelled GCRF, cellulose acetate strips were sprayed with En^{3}Hance spray (New England Nuclear), and then exposed for 7 to 10 days at −70°C to Kodak X-OMAT film (Eastman Kodak Co., Rochester, New York, USA).

Statistics

Data are expressed as mean ± SD. Comparisons were made by two-tailed unpaired Student's *t*-test.

Results

Isolation of GCRF

Triton X-114 partitioning. Unexpectedly, there was no complement inhibitory activity recovered in the lipid phase of

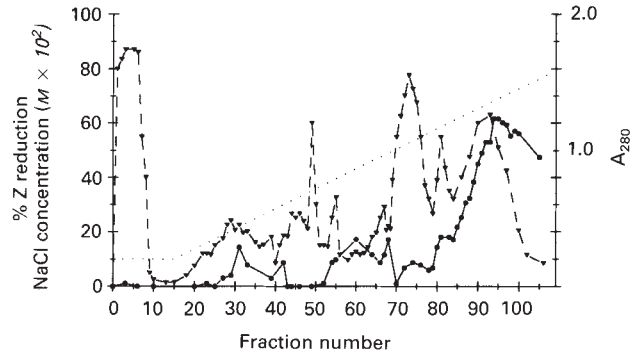

Fig. 1. *Mono Q chromatography of pass-through material from Bio-Rex 70.* Twenty ml was applied, followed by a NaCl gradient from 0.1 to 0.8 M. 0.5 ml fractions were collected and assessed for their ability to decay EC3bBbP, and data expressed as percent z reduction compared to control. Symbols are: (●——●) % z reduction; (▽----▽) A_{280}; (....) NaCl concentration.

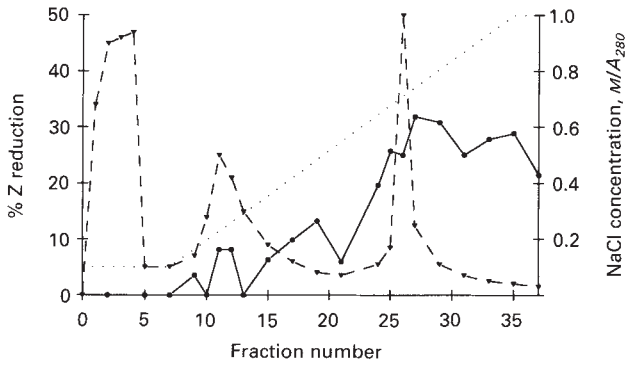

Fig. 3. *Mono Q chromatography of GEC conditioned medium.* After 50 ml of medium was applied, a 30 ml gradient from 0.1 to 1.0 M NaCl was applied, and 1 ml fractions collected and tested for their ability to decay EC3bBbP. Symbols are: (●——●) % z reduction; (▽----▽) A_{280}; (....) NaCl concentration.

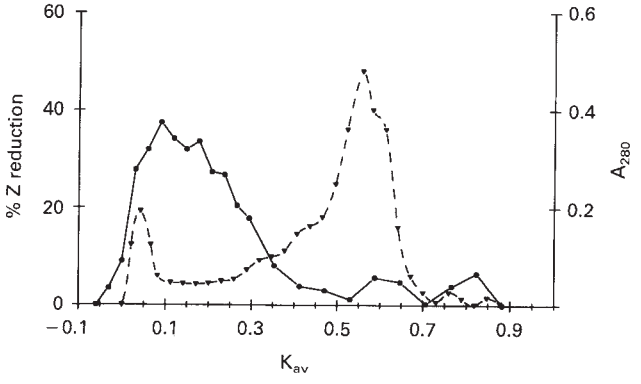

Fig. 2. *Superose 6 chromatography of GEC concentrate from Mono Q.* The bulk of complement inhibitory activity appeared at $K_{av} \leq 0.3$. Symbols are: (●——●) % z reduction; (▽----▽) A_{280}.

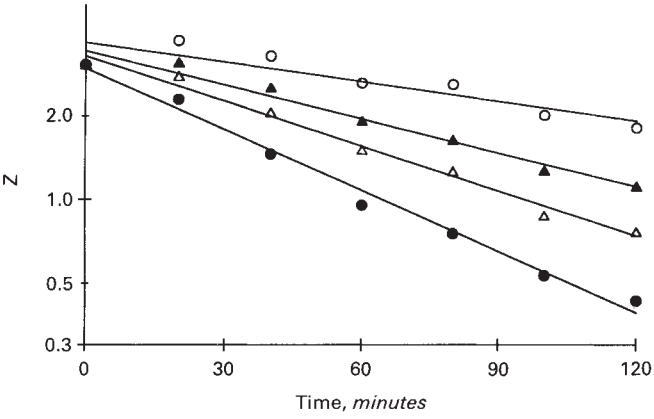

Fig. 4. *Effect of various concentrations of GCRF on the decay of EC3bBbP.* Symbols are: (○) zero; (▲) 0.75 μg/ml; (△) 1.5 μg/ml; (●) 3.0 μg/ml.

Triton X-114 solubilized GEC, while there was substantial activity in the aqueous phase (693 ± 370 U/mg, where 1 U is the reciprocal of the dilution of sample that resulted in a 30% reduction in z compared to EC3bBbP in buffer alone [4]).

Chromatographic purification. To remove potential contaminating CR1, a cation exchange step on Bio-Rex 70 [3, 30] in bis-Tris buffer (pH 6.0) was performed on the aqueous soluble GEC extract, and the pass-through material subsequently studied. Notably, protein eluted from Bio-Rex 70 with 0.5 M NaCl had complement inhibitory activity (in one isolation, 11.6% of total complement inhibitory activity was in this fraction, but was not studied further), which possibly was CR1.

Pass-through material from Bio-Rex 70 was applied to a Mono Q column equilibrated in bis-Tris buffer and then a linear gradient of NaCl applied. As shown in Figure 1, the bulk of complement inhibitory material appeared after 0.6 M NaCl. Fractions 89 to 105 were pooled, diluted 2:1 with bis-Tris buffer, and then reapplied to the Mono Q column. There was no detectable pass-through protein and bound material was eluted with bis-Tris buffer containing 1 M NaCl. Five hundred μl of the concentrated material was applied at 0.2 ml/min to a Superose 6 column equilibrated in PBS, and 0.5 ml fractions collected. As shown in Figure 2, the majority of complement inhibitory activity had a $K_{av} \leq 0.3$. The peak of activity ($K_{av} \leq 0.1$) was pooled and saved for further studies.

Conditioned medium. To determine whether GEC conditioned medium contained complement inhibitory activity, 50 ml of medium was acidified to pH 6.0, filtered, and then passed over a Mono Q column. As with the solubilized cell extracts, complement inhibitory activity predominantly appeared at NaCl concentrations of ≥ 0.6 M (Fig. 3), suggesting that GEC secreted GCRF into the medium.

Hemolytic studies

As shown in Figure 4, GCRF obtained from the Superose 6 column reduced the $t_{1/2}$ of EC3bBbP in a dose-dependent fashion. In buffer alone, the $t_{1/2}$ of EC3bBbP was 128 min, which decreased to 74, 56, and 41 minutes in the presence of 0.75, 1.5, and 3.0 μg/ml of GCRF protein, respectively. If EC3b were preincubated with GCRF for 30 minutes, followed by addition of B, D, and P to make functional C3/C5 convertases (EC3bBbP), significant inhibition in hemolytic activity occurred

Table 1. Effect of GCRF on EC3b

GCRF concentration μg/ml	Z	
	Washed	Unwashed
0	0.88 ± 0.02	1.04 ± 0.05
0.75	1.12 ± 0.14	0.77 ± 0.09[a]
1.5	0.98 ± 0.03	0.56 ± 0.02[b]
3.0	0.94 ± 0.02	0.44 ± 0.04[b]

EC3b at 1×10^8/ml in $DGVB^{++}$ were incubated for 30 min at 30°C with an equal volume of either buffer alone or varying concentrations of GCRF. Cells were then either washed once and resuspended to 5×10^7/ml in $DGVB^{++}$, or used immediately. Factors B, D, and P were added for 9 min at 30°C to form alternative pathway C3/C5 convertases (EC3bBbP), and convertase activity in 200 μl samples determined by addition of 300 μl NRS-EDTA. After 60 min at 37°C, cells were pelleted and hemolysis determined by measuring hemoglobin in the supernatant at OD_{414}. Data are expressed as the average number of hemolytic sites per cell (z), and are the mean ± SD ($N = 3$).

[a] $P < 0.02$; [b] $P < 0.001$ compared to buffer alone

Table 2. Effect of enzymes on GCRF

Enzyme	GCRF	Z
None	+	0.92 ± 0.04
Chondroitinase ABC	+	1.98 ± 0.38[a]
Chondroitinase AC	+	0.99 ± 0.03
Heparitinase	+	0.93 ± 0.05
Streptomyces hyaluronidase	+	0.93 ± 0.08
Neuraminidase	+	1.68 ± 0.05[b,c]
Trypsin	+	1.87 ± 0.05[b,c]
None	−	2.27 ± 0.06[b]

GCRF was exposed to the various enzyme preparations listed for 60 min at 37°C. All contained 5 mM DFP, except for trypsin, which was neutralized after incubation with soybean trypsin inhibitor. One hundred μl EC3bBbP at 1×10^8/ml were incubated with 100 μl of the various digests (diluted 1/25) for 90 min at 30°C, and resulting convertase activity determined by addition of NRS-EDTA as before. Values are mean ± SD ($N = 3$).

[a] $P < 0.01$; [b] $P < 0.001$ compared to GCRF unexposed to enzymes; [c] $P < 0.001$ compared to incubations in the absence of GCRF

only if cells were not washed prior to addition of B, D, and P (Table 1). These results show that GCRF can prevent alternative pathway C3/C5 convertase formation as well as accelerate their decay, but it does not bind to E, either through membrane binding (as DAF does [31]), binding to sulfo-SIAB on the E surface, or through non-specific interactions, nor does it have sufficient affinity for C3b on the surface of EC3b to remain after washing.

Enzyme studies

GCRF isolation. For these studies, partially purified GCRF was isolated as follows. The aqueous phase from Triton X-114 solubilized GEC (in general, from fifty 100 mm culture dishes) was applied to a Mono Q column in bis-Tris buffer, and subsequently a 10 ml gradient of NaCl applied to 0.6 M. The concentration of NaCl was kept at 0.6 M for 6 ml and then raised to 1 M. The majority of complement inhibitory activity was recovered in the 1 M NaCl pool. Collected material was neutralized with 1 M Tris base (1%, vol/vol), and used for subsequent studies.

Hemolytic assays. As shown in Table 2, GCRF isolated in this manner was highly active on EC3bBbP formed with human C3, in that a 1/25 dilution of GCRF incubated in enzyme inhibitors alone (1:1), reduced z by ≈ 60% after 90 minutes (that is, from 2.27 to 0.92). Trypsin treatment of GCRF nearly completely eliminated its activity (Table 2), supporting that it is a protein. Because GCRF was large and anionic as determined by chromatography, consistent with the known characteristics of proteoglycans synthesized by GEC [14–16], additional studies were performed with specific enzymes reactive with the polysaccharide chains of these molecules [27]. Chondroitinase ABC, but not chondroitinase AC, heparitinase, or *Streptomyces* hyaluronidase, significantly reduced activity of GCRF (Table 2), suggesting that it is a dermatan sulfate-containing proteoglycan [27]. Notably, treatment with neuraminidase also significantly reduced GCRF activity (Table 2), and thus, sialic acid residues must be important for the functional integrity of this molecule.

Although the chondroitinase ABC and neuraminidase preparations used in these studies had been tested and found to be free of protease activity by the manufacturer, additional studies were performed in the presence of protease inhibitors of different classes [27] (**Methods**) in addition to DFP used previously. After decay of EC3bBbP in GCRF exposed to enzyme inhibitors alone, z was 1.02 ± 0.11, while in the presence of buffer alone (that is, an identical formulation as the first, only lacking GCRF), z was 2.19 ± 0.22 ($N = 3$; $P < 0.002$), again illustrating the "decay-accelerating" activity of GCRF. Chondroitinase ABC inhibited GCRF activity in a dose-dependent fashion, such that z was 2.20 ± 0.75, 1.40 ± 0.05, and 1.14 ± 0.04, at enzyme concentrations of 0.5, 0.25, and 0.125 U/ml, respectively. Neuraminidase at 0.5 U/ml also significantly inhibited GCRF activity (z = 1.46 ± 0.09; $P < 0.01$). Therefore, these results support the conclusions that GCRF is a chondroitin (dermatan) sulfate proteoglycan containing sialic acid residues.

To exclude a direct effect of the enzyme preparation on the hemolytic assay, EC3b were preincubated with chondroitinase ABC or neuraminidase at the concentrations used in the above experiments, and then made EC3bBbP with B, D, and P, and decayed for 90 minutes in GCRF. After a 90 minute decay, z was 0.80 ± 0.04, 0.83 ± 0.10, and 0.73 ± 0.06 in cells exposed to neuraminidase, chondroitinase ABC, and protease inhibitors alone, respectively ($N = 5$; NS), indicating that the enzymes alone did not reduce decay of EC3bBbP. Notably, higher concentrations of neuraminidase (0.5 U/10^9 EC3b), did modestly increase susceptibility to NRS-EDTA after formation of AP convertases and subsequent decay in $DGVB^{++}$ (z = 0.93 ± 0.04 and 0.79 ± 0.02, in cells exposed to neuraminidase or buffer alone; $N = 4$; $P < 0.001$), presumably due to the removal of sialic acid on the E membrane, which promotes the accessibility of factor H (in NRS-EDTA) to EC3bBbP [32]. Again, in these neuraminidase-treated cells, GCRF effectively decayed EC3bBbP (z = 0.32 ± 0.01).

Chondroitin sulfate B at 50 μg/ml did not accelerate the decay of EC3bBbP (made with human C3b) compared to buffer alone ($t_{1/2}$ = 43 and 42 min, respectively; $r = -0.99$ for both), while the $t_{1/2}$ was 29 min ($r = -0.97$) in GCRF diluted 1/40 (by protein assay ≈ 14 μg/ml). Therefore, the decay accelerating activity of GCRF was not a nonspecific effect of this glycosaminoglycan.

Size exclusion chromatography. As the majority of GCRF's activity appeared at $K_{av} \leq 0.3$ and several of the enzyme

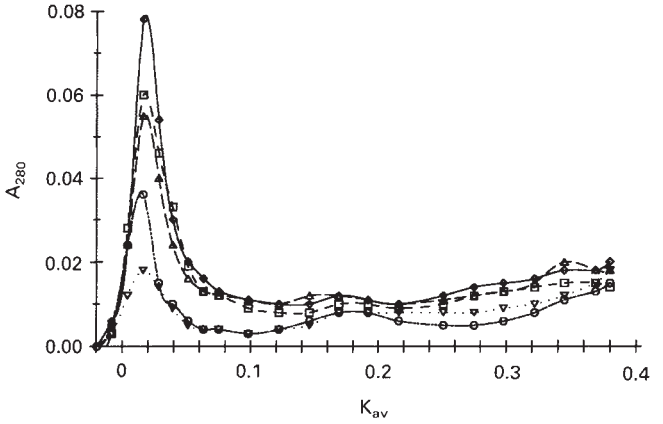

Fig. 5. *Superose 6 chromatography of GCRF following enzyme treatment.* Symbols are: (◇–◇) no enzyme; (□----□) heparitinase; (△----△) chondroitinase AC; (○–––○) chondroitinase ABC; (▽···▽) neuraminidase.

preparations had contaminating/carrier proteins that were smaller, the data presented will be limited to $K_{av} < 0.4$. Chondroitinase ABC and neuraminidase reduced the size of the peak at $K_{av} \approx 0.02$ as well as the protein appearing after this peak (Fig. 5). Chondroitinase AC and heparitinase slightly reduced the early peak compared to incubations in enzyme inhibitor alone, however, they did not affect later appearing protein (Fig. 5). Trypsin also reduced, but did not eliminate, the early peak and subsequently appearing protein (not shown). Therefore, the loss of activity of GCRF following treatment with chondroitinase ABC, neuraminidase, and trypsin, was associated with a reduction in its size.

^{35}S and ^{3}H labelling

GEC were labelled with [^{35}S]sulfate and [^{3}H]leucine for 15 hours, and the medium and cellular extract chromatographed on Mono Q. As shown in Figures 6A and B, the predominant appearance of ^{35}S-labelled material late in the NaCl gradient (≥ 0.6 M) corresponded to activity in hemolytic assays (Figs. 1 and 3), providing further evidence that GCRF is a sulfated proteoglycan. In both the medium (Fig. 6B) and aqueous soluble cell extract (Fig. 6A), the amount of ^{3}H-labelled protein at ≥ 0.6 M NaCl was a relatively minor fraction of total protein.

Size exclusion chromatography with guanidine HCl

GCRF was isolated as described above, only instead of using bis-Tris buffer containing 1 M NaCl to elute GCRF from the Mono Q column following the 0.6 M plateau, buffer containing 0.05 M Tris, 4 M guanidine HCl, pH 7.0 was used instead, to eliminate non-covalent interactions between proteoglycans and proteins [27]. GCRF was then run over a Superose 6 column and fractions tested for hemolytic inhibitory activity. As shown in Figure 7, three distinct peaks of activity were recovered at K_{av} 0.03, 0.26, and 0.38, suggesting that GCRF is a heterogeneous molecule with three apparent species of different sizes.

Additional studies were done with the aqueous phase from Triton X-114 solubilized ^{35}S-labelled GEC that was made 4 M guanidine. Following buffer exchange into 7 M urea, this was applied to a Mono Q column, a gradient of NaCl was run to 0.6 M NaCl, and material remaining on the column eluted with 4 M

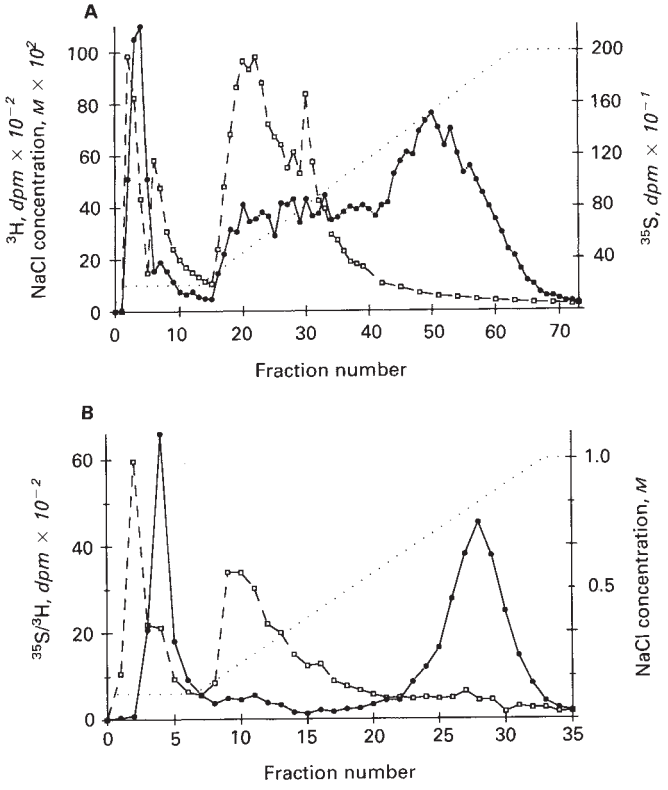

Fig. 6. *Mono Q chromatography of GEC* **(A)** *and GEC conditioned medium* **(B)** *labelled with [^{35}S]sulfate and [^{3}H]leucine.* Once loaded, a 25 ml gradient from 0.1 to 1.0 M NaCl was run, and 0.5 ml (A) or 1.0 ml (B) fractions collected, and ^{35}S and ^{3}H radioactivity determined. Symbols are: (□----□) ^{3}H-dpm/fraction; (●——●) ^{35}S-dpm/fraction; (....) NaCl concentration.

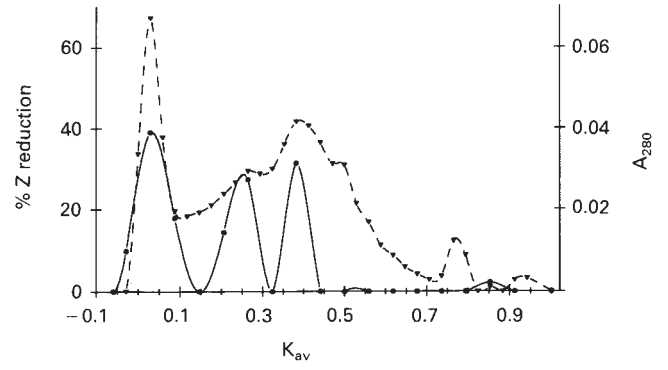

Fig. 7. *Superose 6 chromatography of GCRF under dissociative conditions in 4 M guanidine HCl.* One ml fractions were collected, desalted on a Sephadex G-25 column, and tested for their ability to decay EC3bBbP. Symbols are: (●——●) % z reduction; (▽----▽) A_{280}.

guanidine HCl. Two hundred μl, containing 4.6×10^{4} dpm, was then applied to a Sepharose CL-4B column. As shown in Figure 8, the main peak of ^{35}S-labelled material appeared at K_{av} 0.54 (long arrow), although as with the Superose 6 column, there were peaks of smaller size (K_{av} 0.61 and 0.67; small arrows). The ^{35}S-labelled material eluted from the Mono Q column with 4 M guanidine HCl inhibited EC3bBbP in a dose-dependent fashion ($r = -1.00$ for z values vs. concentration), establishing that GCRF was present in this preparation.

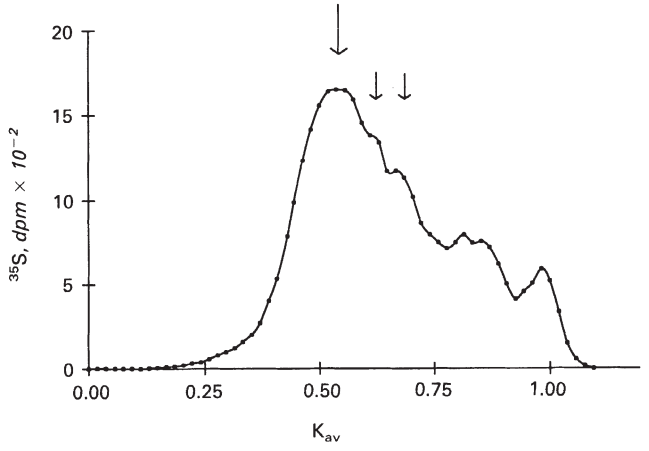

Fig. 8. *Sepharose CL-4B chromatography of ^{35}S-labelled GCRF isolated under dissociative conditions.*

Cellulose acetate electrophoresis

Glycosaminoglycans from ^{35}S-labelled GCRF isolated under dissociative conditions migrated to a single spot (Fig. 9A) with a mobility similar to that of heparan sulfate standard (Fig. 9B). However, chondroitinase ABC, but not heparitinase treatment eliminated the ^{35}S spot (not shown). As unknown glycosaminoglycans do not always migrate equivalently to standards [27], enzyme sensitivity was necessary to establish the identity of the GCRF glycosaminoglycan as being dermatan/chondroitin sulfate. Unlabelled GCRF glycosaminoglycan showed identical mobility to the ^{35}S-labelled GCRF isolated under dissociative conditions (Fig. 10). As with labelled GCRF glycosaminoglycan, heparitinase treatment had no effect, but notably, chondroitinase AC treatment eliminated this spot (Fig. 10), establishing that GCRF glycosaminoglycan contains glucuronic acid residues [27].

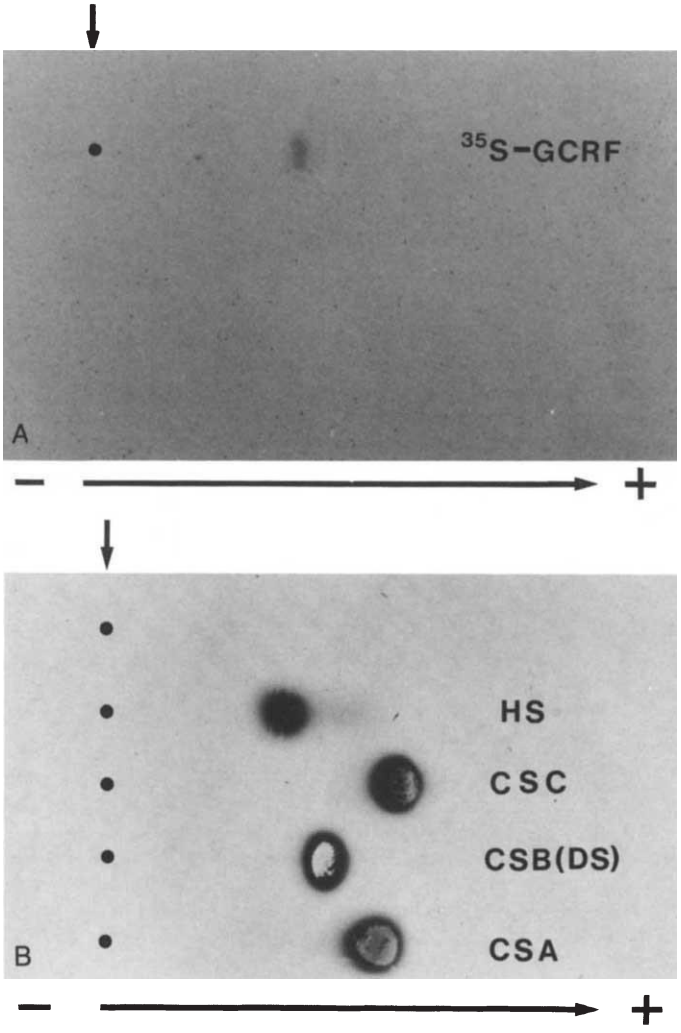

Fig. 9. *Cellulose acetate electrophoresis of ^{35}S-labelled GCRF isolated under dissociative conditions.* **A** is the autoradiogram, while **B** is the same sheet stained with alcian blue to reveal the glycosaminoglycan standards. The origins are indicated by the short arrow and the direction of migration by the long arrow. CSA, chondroitin 4-sulfate; CSB, dermatan sulfate; CSC, chondroitin 6-sulfate; HS, heparan sulfate.

Discussion

This study demonstrates that cultured rat GEC synthesize and secrete into the medium a unique complement regulatory factor, GCRF. That GCRF has complement inhibitory activity is shown by its ability to accelerate the decay of preformed alternative pathway C3/C5 convertases, and also prevent their formation in a dose-dependent fashion. On the basis of its susceptibility to chondroitinase ABC and trypsin digestion, it is a proteoglycan. As digestion with heparitinase, chondroitinase AC, or *Streptomyces* hyaluronidase did not affect its activity, it appears to be a dermatan sulfate proteoglycan [27]. In metabolic labelling studies, substantial ^{35}S was incorporated into macromolecules with the chromatographic characteristics of GCRF, adding further support to its being a sulfated proteoglycan.

As shown by cellulose acetate electrophoresis, chondroitinase AC digested the glycosaminoglycans of GCRF, and therefore GCRF contains glucuronic acid residues; however, the activity of GCRF was unaffected by chondroitinase AC, and thus glucuronic acid may be unnecessary for its functional activity on complement. Alternatively, chondroitinase AC may not have the access to glucuronic acid in the intact GCRF that it has in the isolated glycosaminoglycan. As dermatan sulfate may contain more than half of its uronic acid residues as glucuronic acid (the remainder being iduronic acid) [33], the data presented in this study are consistent with the identification of GCRF as a dermatan sulfate proteoglycan.

Dermatan sulfate glycosaminoglycans alone did not affect alternative pathway C3/C5 convertases, suggesting either that the dermatan sulfate glycosaminoglycans of GCRF have a unique composition, or that the activity of GCRF is due to its three dimensional configuration, which in the case of proteoglycans can be quite complex [34]. Notably, dermatan sulfate proteoglycans may have higher affinity for proteins than other proteoglycans (of equal charge density), perhaps due to their spatial characteristics [34]. Support that the dermatan sulfate glycosaminoglycans of GCRF themselves are not responsible for complement inhibition comes from studies in which the activity of GCRF was substantially reduced after digestion with trypsin, which affects the protein core but not the glycosaminoglycan side-chains. The core protein alone also appears to lack

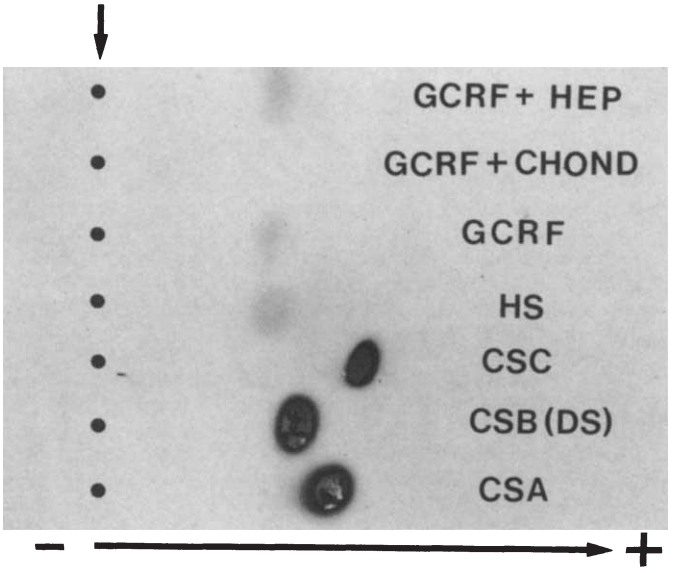

Fig. 10. *Cellulose acetate electrophoresis of GCRF following enzyme digestion.* GCRF was incubated with either heparitinase (GCRF + HEP), chondroitinase AC (GCRF + CHOND), or no enzyme (GCRF). Legends are the same as in Figure 9.

complement inhibitory activity, as digestion with chondroitinase ABC led to complete loss of hemolytic inhibitory activity.

The significant decrease in GCRF's activity following neuraminidase digestion shows that sialic acids are important for its activity. The reduction in size of GCRF by size-exclusion chromatography (Fig. 5), suggests either that these sialic acids are important for the spatial characteristics of GCRF and/or that they occupy internal positions in the polysaccharide side chains [35]. Alternatively, as size exclusion chromatography was performed under non-dissociative conditions, sialic acids may have led to adherence of (cationic) proteins or to self-aggregation of proteoglycans [36].

The finding of a dermatan sulfate proteoglycan from GEC is consistent with two recent studies on cultured human GEC by Thomas et al [15] and by Klein et al [16]. In the latter study, dermatan sulfate proteoglycan, identified by susceptibility to chondroitinase ABC and relative insensitivity to chondroitinase AC, was identified in both the culture medium and cell layer. By anion exchange chromatography, it was the most negatively charged of the proteoglycans, similar to what is described in this study for GCRF. However, under dissociative conditions on Sepharose CL-4B, it appeared as a broad peak centered around K_{av} 0.27 [16], which is larger than GCRF (K_{av} 0.54). Notably, in the study by Klein et al, unlike the other proteoglycans isolated, dermatan sulfate proteoglycan did not bind to a hydrophobic affinity column [16], similar to the case with GCRF (unpublished observations).

The exact mechanism of GCRF's action was not determined in this study, although it acts similarly to the C3b binding proteins, factor H, DAF, and CR1, in that it decays preformed alternative pathway C3/C5 convertases [6]. It has further similarities to DAF in that it prevents the formation of these convertases [6, 31], and it does not have high enough affinity for C3b [5, 37] to allow its purification by C3b affinity chromatography (unpublished observations). However, GCRF does not incorporate into the E membrane as DAF does [31]. As sialic acids are known to increase accessibility of factor H to cell-bound C3b [32], one consideration is that GCRF was increasing the access of factor H in NRS-EDTA to C3b on the E surface. However, the fact that the $t_{1/2}$ of EC3bBbP was decreased by GCRF would suggest that its effect was directly on these convertases rather than through a factor H "cofactor activity" (that is, if the latter were the case, one would expect that the $t_{1/2}$ would not be affected, but rather that the z reduction would be constant at all time points).

Heparin, a highly sulfated glycosaminoglycan, inhibits several steps of complement activation, including the formation of EC3bBbP [38]. Heparin does not, however, accelerate the decay of EC3bBbP [39], and the data presented here show that dermatan sulfate glycosaminoglycan also does not enhance the decay of this intermediate. Notably, squid cartilage chondroitin sulfate E glycosaminoglycan accelerates the decay of EC3bBbP (made with activated P) in addition to preventing its formation [39]. Chondroitin sulfate E proteoglycan isolated from murine bone marrow-derived mast cells also prevents the formation of EC3bBbP; the ability of this proteoglycan to accelerate the decay of EC3bBbP was not determined in this study, however [39]. It appears as though chondroitin sulfate E and GCRF are unrelated, given the ineffectiveness of GCRF glycosaminoglycan to hasten the decay of EC3bBbP and that the uronic acid residues in chondroitin sulfate E appear to be exclusively glucuronic acid [39]. However, determination of the precise uronic acid composition and the sulfation pattern of GCRF requires additional study.

As proteoglycans are an integral component of the glomerular basement membrane [10–13], the findings reported here may have relevance in vivo. A complement regulatory factor in the glomerular basement membrane and the subepithelial space may be of particular importance, given the large size of the fluid phase complement regulatory proteins, C4 binding protein ($M_r \approx 590$ kD [2]) and factor H (although factor H has an M_r of 150 kD, it behaves as a molecule of 300 kD [1], presumably due to its elongated asymmetric shape [40]), while the proteins of the spontaneously active alternative pathway and the terminal complement sequence have $M_r \leq 206$ kD [41]. Because the glomerular basement membrane acts to restrict passage of proteins on the basis of size and charge [10], there may be relative hindrance of these regulatory proteins, while the active complement proteins should be more accessible to sites within the glomerular basement membrane and on the GEC. Hence, the elaboration of a complement regulatory factor by GEC may restrict complement activation that otherwise might occur in the glomerular capillary wall. The lack of high affinity of GCRF for C3b may be important to prevent C3b from accumulating in the glomerular basement membrane. It remains to be seen whether GCRF is like DAF, in having a much higher affinity for the active bimolecular complex of C3bBb, than either protein alone [37], thereby allowing it to be a "selective" complement inhibitor.

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